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Formation of Benzo(a)pyrene/DNA Adducts and Their Relationship to Tumor Initiation in Mouse Epidermis

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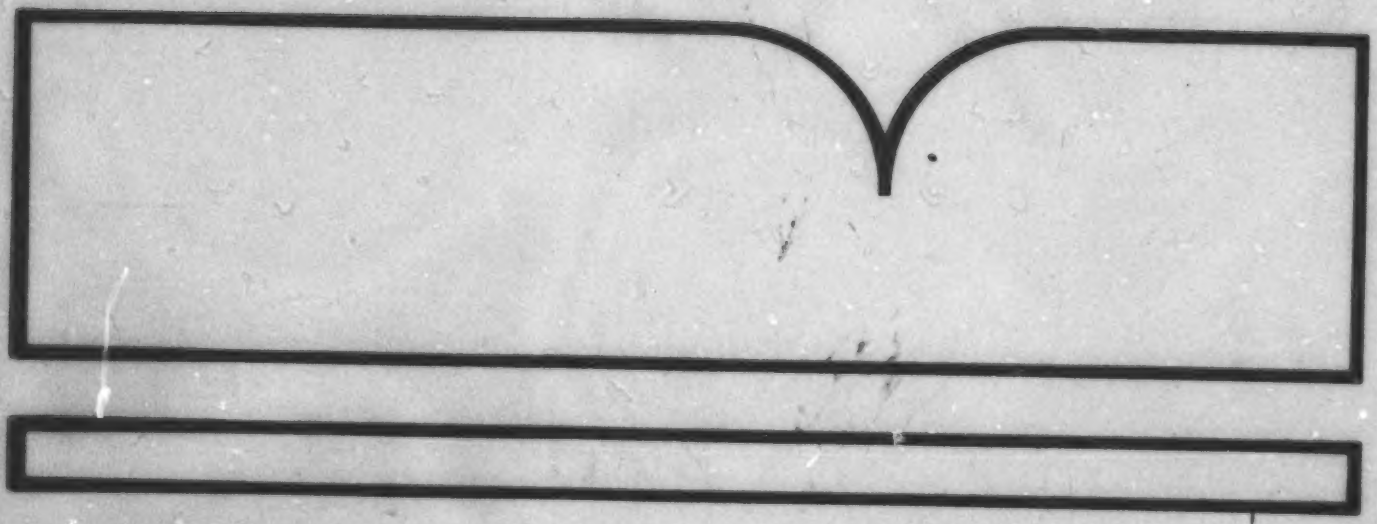

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JOURNAL ARTICLE

Formation of Benzo(*a*)pyrene/DNA Adducts and Their Relationship to Tumor Initiation in Mouse Epidermis[1]

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ABSTRACT

The tumorigenicity of benzo(*a*)pyrene [B(a)P] applied topically as a skin tumor initiator in Sencar mice and the formation of epidermal B(a)P/deoxyribonucleoside adducts were compared over a similar range of doses (50 to 1600 nmol). The tumor-initiating activity of B(a)P, its covalent binding to mouse epidermal DNA, and the formation of the major hydrocarbon/deoxyribonucleoside adduct showed approximately parallel dose-response curves. The major hydrocarbon/deoxyribonucleoside adduct formed cochromatographed with marker adducts of N^2-{10*S*-[7*R*,8*S*,9*R*-trihydroxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene]yl}/deoxyguanosine while other minor adducts also were observed. The disappearance of DNA-bound products in the epidermis was followed for 21 days after an initiating dose of B(a)P (100 nmol) was applied topically to the mice. The half-lives of the B(a)P/deoxyribonucleoside adducts and the total radioactivity bound to the DNA were 4.5 and 5.5 days, respectively. However, in spite of the loss of measurable DNA-bound material, the tumor yield was unchanged regardless of whether promotion was begun 7 or 21 days after initiation. The results suggest a possible causal relationship between B(a)P/deoxyribonucleoside adduct formation and papilloma formation in mouse skin.

INTRODUCTION

PAH[3] are widely distributed in our environment and are implicated in cancer of the skin, scrotum, and bronchus, indicating the importance of studies of their biological fate. Based on extensive evidence accumulated in the last 7 years, it would appear that, in order to exert their mutagenic (5), cell-transforming (20), and tumorigenic or carcinogenic effects (10), PAH must be metabolically activated. For example, an ultimate tumorigenic metabolite of B(a)P, a widely investigated PAH, is believed to be (+)-*anti*-BPDE (7). Recent data have established the potent biological activities of (±)-*anti*-BPDE and (+)-*anti*-BPDE. For example, they are highly active mutagens in both bacterial (30) and mammalian (14, 21) cells, and they are tumor initiators in mouse skin (27) and complete carcinogens in the lungs (16) of susceptible strains of mice. The electrophilic nature of BPDEs is such that they readily alkylate DNA bases and react with phosphate in DNA to produce phosphotriesters which lead to strand scission (11). Many but not all studies with diverse classes of chemical carcinogens have revealed a good relationship between the degree of carcinogenicity and extent of reaction with DNA (18). With the PAH, a good correlation has been observed between the differing carcinogenicities in mouse skin and their covalent binding to DNA but not RNA or protein (6).

Following topical application of B(a)P to mouse skin *in vivo* or treatment of cells or organ explants in tissue culture, the major hydrocarbon-deoxyribonucleoside adduct formed is 7*R*-*anti*-BPDE/*trans*-deoxyguanosine (24). Newbold *et al.* (22) demonstrated that B(a)P-induced cell-mediated mutagenesis in cultured cells was concomitant with the formation of a BPDE/DNA adduct in the treated cells. Other workers have shown that a correlation exists between formation of the 7*R*-*anti* BPDE/*trans*-deoxyguanosine adduct in DNA and mutagenesis in mammalian cells (31).

A recent study using female HA/ICR mice reported a linear dose-dependent binding of B(a)P to mouse epidermal DNA and little if any loss of adducts for 49 hr (23). Another study reported a dose-dependent increase in the binding of 7,12-dimethylbenz(*a*)anthracene to mouse epidermal DNA (26). The current study was undertaken to explore further the relationship between the formation and persistence of hydrocarbon/deoxyribonucleoside adducts and tumor formation in the same animals. To achieve this, an extensive dose response for tumor initiation with B(a)P was compared with the corresponding formation of hydrocarbon/deoxyribonucleoside adducts over a similar dose range. The apparent parallelism in these dose-response curves suggested a possible causal relationship between binding of the carcinogen to DNA and formation of skin papillomas.

MATERIALS AND METHODS

Chemicals. [^{3}H]B(a)P (specific activity, 16 to 56 Ci/mmol) was purchased from the Amersham-Searle Corp., Arlington Heights, Ill., and where necessary diluted with unlabeled B(a)P (Gold Label; Aldrich Chemical Co., Milwaukee, Wis.). [^{3}H]-*trans*-B(a)P-7,8-diol (specific activity, 298 mCi/mmol) was supplied by Midwest Research Institute, Kansas City, Mo. DNase I (EC 3.1.4.5) from bovine pancreas (P. L. Biochemicals, Milwaukee, Wis., or Boehringer-Mannheim Biochemicals, Indianapolis, Ind.), snake venom phosphodiesterase (EC 3.1.5.1) from *Crotalus adamanteus* (Worthington Biochemical Corporation, Freehold, N. J., or Boehringer Mannheim), alkaline phosphatase (EC 3.1.3.1) Type III from *Escherichia coli* (P. L. Biochemicals or Sigma Chemical Co., St. Louis, Mo.), and RNase A (EC 3.1.4.22) (Worthington) were used to hydrolyze the DNA and to remove contaminating

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: PAH, polycyclic aromatic hydrocarbons; B(a)P, benzo(*a*)pyrene; (+)-*anti*-BPDE, (+)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene; (±)-*anti*-BPDE, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene; BPDE, benzo(*a*)pyrene diol epoxide; *trans*-B(a)P-7,8-diol (±)-*trans*-7,8-dihydro-7,8-dihydroxybenzo(*a*)pyrene; TPA, 12-O-tetradecanoylphorbol-13-acetate; HPLC, high-pressure liquid chromatography; ODS, octadecyl silane; 9-OH-B(a)P, 9-hydroxybenzo(*a*)pyrene; (±)-*syn*-BPDE, (±)7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene.

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RNA prior to hydrolysis. TPA was obtained from Chemical Carcinogenesis, Eden Prairie, Minn. All other chemicals used were of the highest purity available.

Tumor Experiments. Groups of at least 30 Sencar mice were used for all tumor experiments. Animals aged 7 to 9 weeks were shaved with surgical clippers 2 to 4 days prior to treatment, and only those mice in the resting phase of the hair cycle were used. B(a)P (50 to 1600 nmol) was applied as a single topical application followed 1 week later by twice weekly applications of 3.4 nmol TPA. In one experiment, treatment with TPA was delayed until 21 days after application of the initiator. The incidence of papillomas was recorded weekly, and the tumors were removed at random for histological verification. All tumor data represent maximal tumor responses.

DNA Binding *in Vivo*. Groups of 30 Sencar mice were treated as in the tumor experiments except that TPA was not used. For the dose-response experiments, groups of animals were treated with various doses of B(a)P (10 to 1600 nmol). Generally, 0.5 mCi of isotope was applied to each animal for the 10- to 800-nmol/mouse doses so that the specific activity varied over the dose range utilized. At doses of 800- to 1600-nmol/mouse, 1 mCi/animal was used so the specific activity enabled adequate detection of DNA adducts. In experiments to determine the persistence of B(a)P/DNA adducts, groups of 30 mice were treated with [^{3}H]B(a)P (100 nmol). Generally, 0.25 mCi radioactivity per animal was used but for the later time points 1 mCi radioactivity per animal was used. Twenty-four hr following application of the hydrocarbon, mice were sacrificed by cervical dislocation, and the epidermal material was removed by the heat treatment method (19). For initial experiments, DNA was extracted by the method of Huberman and Sachs (13). In later experiments, DNA was extracted essentially by the method of Diamond *et al.* (9). The extracted DNA was dissolved to an approximate concentration of 1 mg/ml and quantified spectrophotometrically at 260 nm assuming $E^{1\%}_{1cm}$ = 260. Purity was assessed by the absorbance ratios $A_{260}/A_{280} > 1.86 \pm 0.008$ (S.D.) and $A_{260}/A_{230} > 2.41 \pm 0.01$. The amount of radioactivity associated with the purified DNA samples was measured in aliquots after digestion with DNase I (see below) in a Packard Tri-Carb liquid scintillation counter using ACS II (Amersham-Searle Corp.) scintillation cocktail. Sufficient counts were accumulated for each sample to provide for less than a 10% error with 95% confidence limits.

Analysis of Hydrocarbon/Deoxyribonucleoside Adducts. The DNA was hydrolyzed sequentially using DNase I, snake venom phosphodiesterase, and alkaline phosphatase to give a mixture of hydrocarbon/deoxyribonucleoside adducts, free deoxyribonucleosides, and some undigested DNA, as described previously (4). These were subsequently separated by either HPLC essentially as described by Weinstein *et al.* (28), or Sephadex LH20 chromatography as described by Baird and Brookes (4). Prior to analysis by HPLC, the enzymatic hydrolysates were applied to a short column of Sephadex LH-20 (0.9 x 3 cm) and washed with water (10 ml) to remove unhydrolyzed DNA and unmodified deoxyribonucleosides. The less polar hydrocarbon/deoxyribonucleoside adducts were eluted with methanol (5 ml) and separated by HPLC using an Ultrasphere ODS column connected to an Altex Model 312 liquid chromatograph. Adducts were eluted at ambient temperature using an isocratic methanol/water (1/1) solvent system at a flow rate of 1 ml/min as described previously (1). Fractions (0.5 ml) were collected and analyzed for radioactivity as described above. The B(a)P/DNA adducts were tentatively identified where possible by co-chromatography with known marker adducts prepared as described previously (2, 3). It should be noted that the B(a)P/DNA adducts elute much later from the ultrasphere ODS column and with better separation than shown in our previous investigations with other ODS columns (2, 3).

RESULTS

Dose-Response Relationship for Tumor Initiation with B(a)P. The skin tumor-initiating activities of the different doses of B(a)P in Sencar mice are shown in Table 1. A clear dose-response relationship was observed between the amount of B(a)P applied as an initiator and the number of papillomas observed in the range of 100- to 600-nmol/mouse. At higher doses of B(a)P, the tumor response leveled off. This can be clearly seen in Chart 1 with the results expressed as a log dose-response curve. A maximum tumor yield of approximately 6 papillomas/mouse was observed at initiating doses above 600 nmol. In the linear region, the dose-response plot had a slope of approximately unity.

Dose-Response Relationship for Covalent Binding of [^{3}H] B(a)P to Epidermal DNA. The covalent binding of B(a)P to

Table 1

Dose response for skin tumor initiation with B(a)P in Sencar mice

At least 30 Sencar mice were used for each experimental group. Animals were initiated with various doses of B(a)P and 1 week later received twice weekly applications of 3.4 nmol TPA for 24 weeks.

Doses (nmol)	Papillomas/mouse at 24 weeks[a]	% of mice with papillomas
0[b]	0.1	10
50	1.1	64
100	1.3	70
200	2.9	75
400	4.8	82
600	5.7	94
800	5.7	84
1000	6.0	100
1200	5.9	100
1600	6.0	87

[a] Average number of papillomas per surviving mouse at 24 weeks.
[b] Mice in this group received the acetone vehicle (0.2 ml) at the time of initiation and 1 week later began receiving twice weekly applications of TPA for 20 weeks.

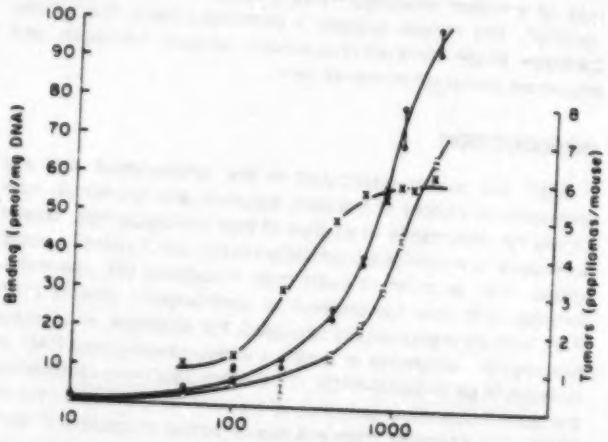

Chart 1. Semi-log plot of the dose-response relationships with B(a)P for tumor initiation and covalent binding to DNA in mouse epidermis. For the tumor initiation experiments, B(a)P was applied (at various doses to the skins of mice) as a single topical application. One week after initiation, mice received twice weekly applications of 3.4 nmol TPA for 24 weeks. For the binding experiments, [^{3}H]B(a)P was applied at various doses, and mice were sacrificed 24 hr later. The epidermal DNA was isolated and analyzed as described under "Materials and Methods." ×, average number of papillomas per mouse at 24 weeks of promotion; ●, total covalent binding of [^{3}H]B(a)P metabolites to epidermal DNA expressed as pmol/mg DNA; and □, *anti*-BPDE bound to deoxyguanosine expressed as pmol/mg DNA. The specific activity of [^{3}H]B(a)P used for each dose was as follows: 10 nmol, 1.1×10^5 dpm/pmol; 50 nmol, 2.2×10^4 dpm/pmol; 100 nmol, 1.1×10^4 dpm/pmol; 200 nmol, 5.5×10^3 dpm/pmol; 400 nmol, 2.75×10^3 dpm/pmol; 600 nmol, 1.83×10^3 dpm/pmol; 800 nmol, 2.75×10^3 dpm/pmol; 1000 nmol, 2.2×10^3 dpm/pmol; and 1600 nmol, 1.38×10^3 dpm/pmol.

epidermal DNA was monitored over a dose range similar to that used in the tumor experiments. Interestingly, a dose-dependent increase in gross covalent binding of B(a)P to DNA was observed with a slope of 0.89. This value, obtained from the double-logarithmic plot shown in Chart 2, is in agreement with the value reported by Pereira *et al.* (23). In order to determine the amounts and nature of the hydrocarbon/deoxyribonucleoside adducts formed at the different doses, the DNA samples were hydrolyzed as described above. The methanol-soluble material containing the hydrocarbon adducts, which represented 70.56 ± 4.38% of the total DNA-bound radioactivity, was analyzed by HPLC. Elution profiles of epidermal DNA samples from mice treated 24 hr previously with the varying doses of B(a)P were qualitatively similar. A typical profile of the adducts observed following application of 200 nmol B(a)P is shown in Chart 3*a*. In agreement with our previous results with the mouse skin system (2) and those of others (12, 17), the major hydrocarbon/deoxyribonucleoside adducts (Peak IV) cochromatographed with the major adduct formed following reaction of (±)*anti*-BPDE with DNA (Chart 3*b*, *Peak C*) and which others have characterized as 7*R*-*anti*-BPDE/*trans*-deoxyguanosine. This adduct made up approximately 65.4% of the total methanol-soluble material chromatographed in Chart 3*a*. Smaller amounts of radioactivity (~7.8% of the total methanol-soluble material) eluted around fraction 44 (Chart 3*a*, *Peak I*), and it was demonstrated previously that this material co-chromatographed with deoxyribonucleoside adducts formed following further metabolism of 9-OH-B(a)P (1). This material was also found in our present study to cochromatograph with a 9-OH-B(a)P/DNA-adduct marker prepared from a rat liver microsomal incubation as described previously (1). The amount of material eluting as 7*R*-*anti*-BPDE/*trans*-deoxyguanosine showed a marked dose dependency (Chart 2), exhibiting a linear dose-response relationship over the dose range used with a slope of 0.89. The amount of material eluting as the 9-OH-B(a)P adduct also showed a marked dose dependency with a slope of 1.0 over the dose range used (data not shown).

In addition to *Peaks I* and *IV*, a number of other DNA adducts were routinely observed in mouse epidermis depending on the level of binding and the specific activity of B(a)P utilized. *Peak II* (~2.3% of total; Chart 3*a*, Fraction 58) did not cochromatograph with any of the bound products formed following reaction of (±)-*anti*- or (±)*syn*-BPDE with calf thymus DNA. *Peak III* (~3.2% of total), however, routinely cochromatographed with one of the bound products obtained by reacting (±)-*anti*-BPDE with calf thymus DNA (Chart 3*b*, Peak A). *Peaks V* (~11.9% of

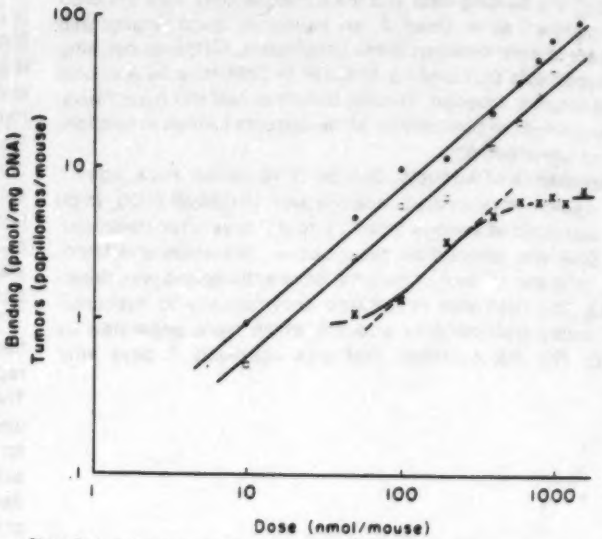

Chart 2. Log-log plot relating the log dose of B(a)P and either the log papillomas per mouse or the log pmol mg DNA for total binding. Also shown is the linear transformation for *anti*-BPDE bound to deoxyguanosine. The symbols are the same as in Chart 1.

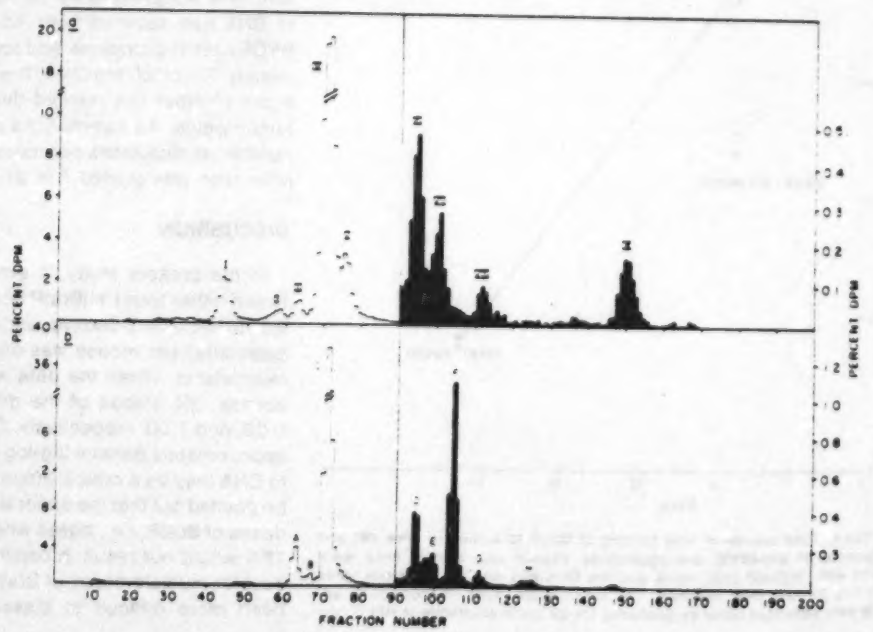

Chart 3. Typical HPLC profile of B(a)P-DNA adducts formed in mouse epidermis. [^{3}H]B(a)P was applied topically at a dose of 200 nmol, and mice were sacrificed 24 hr later. The isolated DNA was hydrolyzed to deoxyribonucleosides and analyzed by HPLC. *a*, B(a)P/DNA adducts formed in mouse epidermis; *b*, DNA adduct markers formed after reacting (±)*anti*-BPDE with calf thymus DNA *in vitro*. □, use left scale of percentage of dpm; ■, use right scale of percentage of dpm.

total) and *IX* (~0.75% of total) cochromatographed with bound products obtained following reaction of (±)-*syn*-BPDE (profile not shown). Of the 4 minor peaks (Peaks VI to IX), *Peaks VI* (~1.75% of total), *VII* (~1.2% of total), and *VIII* (~0.3% of total) cochromatographed with 3 of the 4 deoxyadenosine adducts formed following reaction of (±)-*anti*-BPDE with calf thymus DNA (Chart 3b, *Peaks D, E,* and *G*) (15). Due to the relatively minor quantities or difficulties encountered in quantification, we have not determined the dose-response relationships for these additional peaks. Our results, however, do not preclude the possibility that linear dose-response relationships exist for these peaks as well.

When the binding data and the tumorigenicity data are plotted together, as in Chart 2, an extremely good relationship appears to exist between these parameters, further supporting the hypothesis that binding of B(a)P to DNA may be a critical event in tumor initiation. In order to further test this hypothesis, we examined the persistence of the adducts formed in relationship to tumorigenicity.

Persistence of Adducts. Groups of 30 Sencar mice, aged 7 to 9 weeks, were treated topically with [^{3}H]B(a)P (100 nmol) and sacrificed at various times up to 21 days after treatment. The DNA was isolated as described in "Materials and Methods," and the amount of material covalently bound was determined. The DNA was hydrolyzed enzymatically to hydrocarbon/deoxyribonucleoside adducts which were separated by HPLC. The major adduct that was observed 7 days after treatment with [^{3}H]B(a)P cochromatographed with 7*R*-*anti*-BPDE/*trans*-deoxyguanosine as illustrated in the profile of B(a)P/DNA adducts obtained 24 hr after treatment (Chart 3a) and shown previously (2). In order to further substantiate the nature of the radioactive material eluting with this major peak, Sencar mice were treated topically with [^{3}H]-*trans*-B(a)P-7,8-diol (100 nmol) and sacrificed 24 hr later. The DNA was extracted and hydrolyzed, and the hydrocarbon/deoxyribonucleoside adducts were separated by HPLC. Only one major adduct was observed, and this cochromatographed with the major adduct shown in Chart 3*a*, giving further support that this adduct is derived from a metabolite of *trans*-B(a)P-7,8-diol.

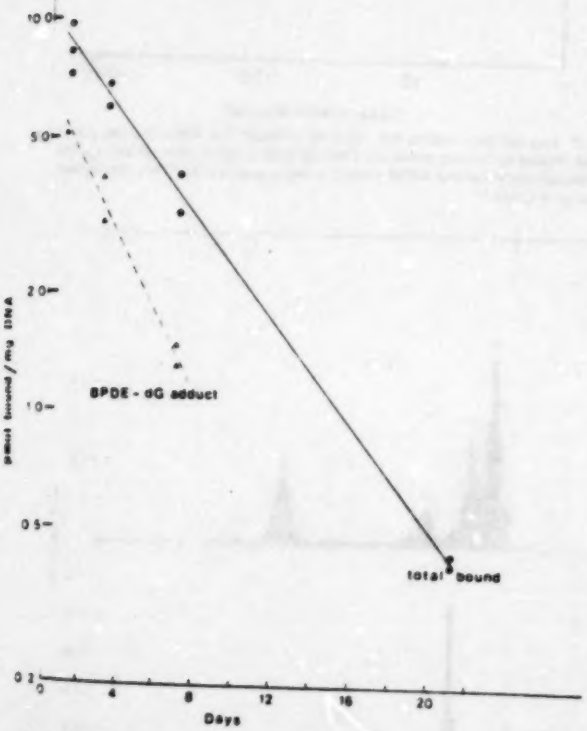

Chart 4. Time course of total binding of B(a)P to epidermal DNA (●) and persistence of *anti*-BPDE/deoxyguanosine adducts (▲). Sencar mice were treated with [^{3}H]B(a)P (100 nmol), and the DNA was isolated at various times from 1 to 21 days after treatment. The hydrocarbon/deoxyribonucleoside adducts were separated either by Sephadex LH-20 chromatography or HPLC.

Table 2

Effect of delayed promotion on tumor initiation with B(a)P in Sencar mice

Thirty mice were used for each experimental group. Mice were initiated with 100 nmol B(a)P and 7 or 21 days later received twice weekly applications of 3.4 nmol TPA for 24 weeks.

Time of starting promotion (days)	Papillomas/mouse[a]	% of mice with papillomas	B(a)P (pmol bound/mg DNA)[b]
7	2.6	81	3.5
21	2.4	76	0.4

[a] Average number of papillomas per surviving mouse at 24 weeks.

[b] pmol [^{3}H]B(a)P covalently bound per mg epidermal DNA 7 or 21 days after topical application of 100 nmol to the skins of mice. Thirty Sencar mice were used for each experimental group. See "Materials and Methods" for further details.

The amounts of the BPDE deoxyguanosine adducts, quantified from both HPLC and Sephadex LH-20 column chromatography, were also plotted against time as illustrated in Chart 4. The decline in the amounts of adducts paralleled closely the decrease in total binding to DNA (Chart 4). The estimated $t_{1/2}$ for total B(a)P bound to DNA and for the BPDE/deoxyguanosine adduct were 5.5 and 4.5 days, respectively. Twenty-one days after application of the 100-nmol dose of B(a)P, quantities of total covalently bound B(a)P and the BPDE/deoxyguanosine adduct had decreased very markedly, *i.e.*, to 0.43 pmol bound per mg DNA and an estimated 0.1 pmol bound per mg DNA, respectively (Chart 4). Thus, from Day 7, when tumor promotion with TPA would normally be started, to Day 21, gross binding to DNA had declined from 3.5 to 0.4 pmol/mg DNA and the BPDE/deoxyguanosine adduct had fallen from 1.4 to approximately 0.1 pmol/mg DNA. It was, therefore, decided to investigate whether this marked decrease in adducts would affect tumor yields. As summarized in Table 2, no difference in the number of papillomas per mouse was observed whether tumor promotion was started 7 or 21 days after tumor initiation.

DISCUSSION

In the present study, a similarity in dose dependence between either total [^{3}H]B(a)P bound to epidermal DNA or the 7*R*-*anti*-BPDE/*trans*-deoxyguanosine adduct and the number of papillomas per mouse was observed, suggesting an apparent relationship. When the data were plotted as log-log dose response, the slopes of the different sets of data were 0.89, 0.89, and 1.00, respectively. One possible interpretation of the approximately parallel log-log plots is that the binding of B(a)P to DNA may be a critical interaction in tumor initiation. It should be pointed out that the tumor data were obtained using initiating doses of B(a)P, *i.e.*, doses which without further treatment with TPA would not result in papilloma formation. If a single, large dose or multiple doses of B(a)P were given, then it would have been more difficult to dissect out those effects related to

initiation from other effects related to promotion.

Chart 1 shows that the tumor response is saturated at doses of B(a)P greater than 600 nmol while no such response is apparent with the binding data, as observed by others (23). One possible explanation of these data, assuming DNA to be the critical target molecule, is that only certain sites in DNA, when modified by the carcinogen, lead ultimately to the formation of tumors and that other sites which can also react with the electrophilic metabolites are unimportant in this respect. Another possibility is that there are subpopulations of epidermal cells with different sensitivities to initiation and that the binding at higher doses of B(a)P (*i.e.*, above 600 nmol) represents binding to DNA in some noncritical cell population.

In the classical 2-stage model of tumor formation in mouse epidermis, it has long been known that application of the promoter can be delayed for up to 1 year after treatment with the initiator before a clear fall in tumor response is observed (25). In the present study, the tumor response was identical whether tumor promotion was begun 7 or 21 days after the animals were initiated with 100 nmol B(a)P (Table 2), while both the total binding to DNA and the amount of BPDE/deoxyguanosine adducts had declined markedly over this time period (Chart 4). These results suggested that the actual level of binding at the time of promoter treatment did not correlate with tumor formation. However, the data presented in Charts 1 and 2 suggested that a causal relationship does exist between tumor formation and DNA binding or hydrocarbon/DNA adduct formation over a wide range of B(a)P doses. Other workers have shown that good relationships exist between the ability of a carcinogen to alkylate or arylate DNA and its potency as a tumorigenic agent (18). Generally, agents which produce a large extent of DNA modification are potent carcinogens (18). Furthermore, tumorigenesis by B(a)P in mouse skin can be inhibited by stopping the formation of hydrocarbon/deoxyribonucleoside adducts even though gross binding to DNA remained the same (8), indicating the importance of investigating the individual hydrocarbon-base adducts formed and not simply the total DNA-bound material. Also, many workers have shown that the formation of hydrocarbon/deoxyribonucleoside adducts occurs when both animal and human cells are mutated by PAH (22, 29, 31).

Several possible mechanisms (among others) could explain the lack of correlation between the level of binding at the time tumor promotion is started and the final tumor response. (*a*) The actual level of adduct formation required to initiate a cell is unknown. Therefore, we cannot say with certainty that the level of binding at Day 21 (shown in Table 2) is below a critical level. (*b*) The heritable change in the DNA may occur early when the carcinogen/DNA adduct is removed and/or the DNA is replicated. Little information is available regarding DNA repair processes in mammalian cells; however, Yang *et al.* (31) showed that in human fibroblasts the excision of lesions caused by (±)-*anti*-BPDE is error-free since the cells recover from cytotoxic and mutagenic lesions caused by the hydrocarbon metabolite. If the adduct persistence data shown in Chart 4 is extrapolated to a period of 1 year (assuming the same rate of loss of adducts), very little material would remain bound to the DNA, approximately 8.3×10^{-25} pmol/mg DNA. This is approximately 5×10^{-15} pmol/cell, assuming 6 pg of DNA per cell, and on this basis the interaction would have to be very specific if it involved the carcinogen/DNA adduct. It is possible that a very small subpopulation of epidermal cells contains the carcinogen/DNA adduct(s) even after a year but, because total epidermis is used to determine the presence of adducts, under these conditions a small subpopulation of cells with carcinogen adducts would go undetected. It is clear that studies of DNA repair in mouse epidermis as well as B(a)P binding and DNA repair in subpopulations of epidermal cells are required to answer these questions.

The present work has produced evidence for and against a relationship between DNA modification and tumor initiation. It is possible that other as yet unknown factors may be important in tumor initiation and that more sensitive techniques are required to further probe the covalent interaction of carcinogens and DNA.

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